

Analysis of polymorphisms in the *SRD5A2* gene and semen parameters in Estonian men

Running head: *SRD5A2* polymorphisms and semen characteristics

Maire Peters^{1,2}, Merli Saare¹, Tanel Kaart³, Kadri Haller-Kikkatalo^{1,4}, Ave Kris Lend⁵, Margus Punab⁶, Andres Metspalu^{2,5,7}, Andres Salumets^{1,5,8}

¹Department of Obstetrics and Gynecology, University of Tartu, L. Puusepa 8, Tartu 51014, Estonia; ²Estonian Biocentre, Riia 23b, Tartu 51010; ³Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Kreutzwaldi 1, Tartu 51014; ⁴Department of Immunology, Institute of General and Molecular Pathology, Centre of Molecular and Clinical Medicine, University of Tartu, Ravila 19, Tartu 51014; ⁵Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Riia 23, Tartu 51010; ⁶Andrology Unit of Tartu University Hospital, L. Puusepa 1a, Tartu 50406; ⁷Estonian Genome Project, University of Tartu, Tiigi 61b, Tartu 50410; ⁸Competence Centre on Reproductive Medicine and Biology, Tiigi 61b, Tartu 50410

Correspondent author: Maire Peters, Department of Obstetrics and Gynecology, University of Tartu, L. Puusepa 8, Tartu 51014, Estonia. Phone: +372 7375882; Fax: +372 7420286; E-mail: mpeters@ebc.ee

This research was supported by Targeted Financing from Estonian Government: SF0180142, SF0180044s09 and PBGMR07903, by the Estonian Science Foundation (Grants 6498 and 6585) and European Union through the European Regional Development Fund in the frame of Centre of Excellence in Genomics and 7 FP Project ECOGENE.

ABSTRACT

Spermatogenesis is an androgen-dependent process and polymorphisms in genes encoding androgen-metabolizing enzymes may be associated with impaired male fertility. The enzyme steroid 5 α -reductase converts testosterone into dihydrotestosterone. We analyzed genotype frequencies of five single nucleotide polymorphisms (SNPs 1-5) (rs632148, rs523349, rs2300701, rs2268797, and rs12470143) in the steroid 5 α -reductase type 2 gene (*SRD5A2*) in 132 azoospermic or oligozoospermic and 211 normozoospermic men. We found no association between investigated genotypes and the occurrence of male infertility. Linear regression analysis revealed a significant correlation between certain alleles of SNP1 and SNP5 and testicular volume among control men. Normozoospermic men carrying the minor allele of all but SNP5 polymorphism exhibited a significantly higher proportion of progressively motile spermatozoa, compared with major homozygotes. However, *SRD5A2* genotypes did not influence sperm concentration, serum testosterone, or follicle-stimulating hormone levels in controls. In conclusion, our results suggest that polymorphisms examined in *SRD5A2* exhibit no adverse effect on semen parameters in Estonian men.

Key words: male infertility, sperm motility, testicular volume, V89L

Introduction

Steroid 5 α -reductase is a key enzyme involved in testosterone metabolism that converts testosterone into the more active metabolite, dihydrotestosterone (DHT). During male development, DHT is necessary for differentiation of the prostate and external genitalia. In post-puberty, DHT is the main androgen responsible for spermatozoan maturation in the epididymis (Robaire and Viger 1995). Human 5 α -reductase activity is provided by type 1 (*SRD5A1*) and type 2 (*SRD5A2*) isozymes (Jenkins et al, 1991; Labrie et al, 1992), which exhibit partially overlapping expression patterns. Both isozymes are expressed in the prostate (Berthaut et al, 1997) and in the adult epididymis but levels of type 2 isozyme are higher (Mahony et al, 1998). However, *SRD5A1* is expressed mainly in skin, while *SRD5A2* is the only form detected in the male external genitalia and seminal vesicles (Thigpen et al, 1993; Eicheler et al, 1994). In addition, testicular 5 α -reductase activity has been detected in spermatogonia (Eicheler et al, 1994) and intact seminiferous tubules (Payne et al, 1973). Thus, 5 α -reduced activity has been presumed to be necessary for maintenance of normal spermatogenesis (Payne et al, 1973).

The *SRD5A2* gene is on chromosome 2 and is composed of 5 exons (Labrie et al, 1992; Thigpen et al, 1992). Several studies have shown that the mutations reducing or eliminating enzymatic activity of *SRD5A2* cause deficient virilization of the male external genitalia or even pseudohermaphroditism (Can et al, 1998; Chavez et al, 2000; Fernandez-Cancio et al, 2004). The majority of these cases involve homozygous missense mutations, which lead to severe masculinization defects (Vilchis et al, 2000; Mazen et al, 2003). Although most of the affected individuals are oligozoospermic or azoospermic, normal fertility has also been reported in men without cryptorchidism (reviewed in Imperato-McGinley and Zhu 2002). Interestingly, a previous study of mutation rates in patients affected by azoospermia and oligozoospermia failed to detect mutations in the *SRD5A2* coding region (Hines et al, 1999). However, it remains possible that common polymorphisms in the *SRD5A2* gene could contribute to male infertility.

Several missense polymorphisms in the *SRD5A2* gene have been shown to affect enzyme activity (Makridakis et al, 2000). The most extensively examined single nucleotide polymorphism (SNP) involves a valine to leucine substitution at codon 89 (V89L). This alteration causes an approximately 30% reduction in enzyme activity in Asian populations (Makridakis et al, 1997) but not in men of European origin (Allen et al, 2001; Hayes et al, 2007). In contrast, a less common missense polymorphism at codon 49, which substitutes an alanine with a threonine at codon 49 (A49T), leads to greatly enhanced enzyme activity (Ross et al, 1998). Polymorphisms altering other amino acids of *SRD5A2*, however, are detected at a very low frequency (Makridakis et al, 2000).

Recently, a new SNP tagging method has been developed that enables description of the all common genetic variation of a gene within a population with greatly reduced SNP genotyping (Johnson et al, 2001). Here, we investigated the frequency distribution of five tagging SNPs (tagSNPs) representing genetic variation across the entire *SRD5A2* gene in 132 patients with idiopathic azoospermia or oligozoospermia. For comparison, we used 211 control normozoospermic men, to enable identification of associations between genetic variations in the *SRD5A2* gene and male infertility. Additionally, the relationships between polymorphisms in *SRD5A2* and fertility parameters in normozoospermic Estonian men were evaluated.

Materials and Methods

Study subjects

A total of 132 infertile men, with the infertility period of more than 12 months, visiting Andrology Unit of Tartu University Hospital in 2005 and 2006 were enrolled in this study. The mean age of patients was 31.8 ± 5.6 (SD) years and they were diagnosed either with non-obstructive azoospermia ($n = 36$) or oligozoospermia ($n = 96$, with a sperm count of $0.01 - 14.0 \times 10^6/\text{mL}$). As a control group, 211 men (18.7 ± 1.6 years of age) under compulsory medical examination for military service (Jensen et al, 2004) were used. All study participants underwent medical examination which included determination of testicular size using Prader orchidometer, measurements of serum follicle-stimulating hormone (FSH) and testosterone (T) levels by chemiluminescence immunoassay (Immulite 2000; DPC, Los Angeles, CA, USA), and semen analysis according to the guidelines of World Health Organization (WHO 1999). Men with known medical reasons for their infertility as pathologies of the epididymis or vas deference, cryptorchidism, mumps, varicocele, retrograde ejaculation, chromosomal abnormalities and Y chromosome microdeletions were excluded from the study. Diagnosis of obstructive and non-obstructive azoospermia was performed using open testicular biopsy. This study was approved by the Tallinn Medical Research Ethics Committee, and written informed consent was obtained from all study participants.

The mean sperm concentration (\pm SD) was $1.8 \pm 2.5 \times 10^6/\text{mL}$ among all patients and $149.2 \pm 88.0 \times 10^6/\text{mL}$ among controls (t -test, $P < 0.001$). Abstinence from ejaculation was 4.0 ± 1.6 days in the patient group and 5.2 ± 2.4 days in controls (t -test, $P < 0.001$). Mean testicular volume was markedly smaller (18.7 ± 7.1 mL) among patients than in controls (26.6 ± 4.9 mL, t -test, $P < 0.001$). Sperm motility parameters were collected from 90 oligozoospermic patients and 209 controls (mean progressive motility $20.0 \pm 17.4\%$ and $57.8 \pm 9.9\%$, respectively. $P < 0.001$).

SNP selection

The pair-wise algorithm of the Tagger program was used to select tagSNPs (de Bakker et al, 2005). Five tagSNPs across the 56 kb of the *SRD5A2* gene were selected using Caucasian population data available in the HapMap database (<http://www.hapmap.org>). An r^2 of 0.9 was selected as a threshold (the mean r^2 of captured alleles was 0.96), enabling prediction of genotypes for 17 SNPs. SNPs with low frequency ($< 5\%$) were excluded, as these variants could not be accurately captured with tagSNPs (Montpetit et al, 2006). TagSNP1 (rs632148) is located

within the 5' UTR of the *SRD5A2* gene, tagSNP2 (rs523349) in exon 1, conferring an amino acid change V89L and remaining tagSNPs (tagSNP3 - rs2300701, tagSNP4 - rs2268797, and tagSNP5 - rs12470143) were located in intron 1 (Figure).

Genotyping

Genomic DNA was isolated from the peripheral EDTA-blood using a salting-out method (Aljanabi and Martinez 1997). PCR was carried out in a total volume of 15 μ l, with 50 ng DNA, 0.2 mM dNTPs, 2.5 mM MgCl₂, 1 \times PCR buffer (Solis BioDyne, Tartu, Estonia), 10 pmol primers, and 0.8 U Hot Start thermostable DNA polymerase HOT FIREPol (Solis BioDyne). Reaction mixtures were preheated (95°C, 10 min), followed by 35 cycles of amplification (95°C for 30 s, 51-63°C for 30 s, and 72°C for 30 s). Restriction fragment length polymorphism analysis was performed to detect polymorphic variants of tagSNPs. PCR primer sequences and restriction enzymes (MBI Fermentas, Vilnius, Lithuania) used are listed in Table 1. PCR products, as well as the restriction fragments, were detected on 2% agarose gel containing 10 μ g/mL ethidium bromide and were visualized by UV transillumination.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corp., Redmond, WA, USA). A *t*-test was used to compare fertility parameters between study groups. Genotypes at each SNP were tested for Hardy-Weinberg equilibrium (HWE) using the χ^2 test. The association between the *SRD5A2* genotypes and male infertility was tested with the χ^2 test, as the effects of potential confounding factors estimated by generalized linear models with logistic link function were absent and statistically not significant. The general linear models incorporating the effects of age and length of abstinence were used to describe the associations between the *SRD5A2* genotypes and fertility parameters (sperm count and motility, serum hormones, and testicular volume). P values < 0.05 were considered statistically significant. Haploview 4.0 (<http://www.broad.mit.edu/mpg/haploview>) program was used to calculate linkage disequilibrium (LD) between each pair-wise combinations of all SNPs (Barrett et al, 2005). The linkage disequilibrium coefficient (*D'*) and *r*² were used to indicate the strength of LD. Haplotypes were inferred using program PHASE 2.1 (Stephens et al, 2001).

Results

SRD5A2 genotype frequencies among study groups

The genotype frequencies of five tagSNPs in the *SRD5A2* gene were determined for 132 infertile patients and 211 normozoospermic control individuals. The distribution of genotypes was consistent with HWE for cases and controls, and for cases and controls combined (all $P > 0.2$). We examined the distribution of *SRD5A2* genotypes, by comparing the number of individuals homozygous for the major allele and the number of those carrying at least one copy of the minor allele (Table 2). We found no associations between the genotypes and the risk for male infertility. Moreover, factoring in potential confounding parameters, such as patient age, length of abstinence, or degree of the severity of the fertility impairment (azoo or oligozoospermia) also failed to reveal a link between genotype and infertility (data not shown).

The results from linkage disequilibrium analysis showed that D' for 5 tagSNPs investigated ranged from 0.45 to 0.95, with the strongest LD observed between SNP1 and SNP2 ($D' = 0.95$), forming a single haplotype block (Figure). Haplotype analysis predicted the presence of 4 major haplotypes (of 25 inferred haplotypes), with a prevalence of over 5% accounting for 82% of all haplotypes (Table 3). Although the frequency of the most common haplotype 1 was higher among controls, while haplotype 2, composed of minor alleles of all studied tagSNPs except SNP5, was more frequent in infertile patients, the differences were not statistically significant.

SRD5A2 genotypes and clinical parameters among normozoospermic controls

Linear models adjusted by age showed that the SNP1 C-allele (GC+CC *versus* GG genotype) and SNP5 T-allele (CT+TT *versus* CC genotype) were associated with smaller (25.8 *versus* 27.3 mL, $P = 0.024$) and larger (27.0 *versus* 25.4 mL, $P = 0.036$) mean testicular volumes, respectively (Table 4). Linear models adjusted by age and the length of abstinence revealed that the proportion of progressively motile category A and B type of spermatozoa was significantly higher among subjects carrying at least one copy of the minor allele (variant homozygotes and heterozygotes) for all polymorphisms investigated, except SNP5 (mean 59-60% *versus* 56% for wild-type homozygotes, $P = 0.009, 0.010, 0.008$ and 0.010 for SNP1 to 4, respectively). The inverse trend was observed for the proportion of immotile (category D) spermatozoa ($P = 0.016, 0.018, 0.008$ and 0.005 for SNP1 to 4, respectively). However, no marked relationship between the *SRD5A2* genotype and non-progressive category C type motility was observed among normozoospermic men (Table 4). In addition, our results indicated a lack of correlation between genotypes

investigated and semen volume, sperm count, and serum hormones (T and FSH) (data not shown).

Discussion

We examined the possible role of 5 common *SRD5A2* gene SNPs in predisposition to male infertility, and their association with semen parameters. Our results indicate that normozoospermic men carrying at least one minor allele of all but SNP5 polymorphism had a significantly higher proportion of progressively moving spermatozoa, while all other semen and hormonal parameters were independent of genetic variation examined at the *SRD5A2* locus. In addition, we found no genetic link between *SRD5A2* and severe male infertility due to azoospermia or oligozoospermia.

Analysis of the role of 5 α -reductase in spermatogenesis has been limited. Recent studies have shown that use of dutasteride and finasteride (a dual inhibitor of type 1 and type 2 enzyme and a specific inhibitor of type 2 enzyme, respectively) suppresses serum DHT levels significantly, while having only a mild (Amory et al, 2007) or no effect on sperm concentration (Overstreet et al, 1999). Some study participants responded to treatment with 5 α -reductase inhibitors, by exhibiting around a 10% reduction in total sperm count. Although not formally demonstrated, genetic differences have been assumed to be one of the most likely reason for this variable inter-subject response (Amory et al, 2007). Furthermore, treatment of fertile men with testosterone enanthate in a male contraceptive study led to development of azoospermia in 50-70% of patients, while the rest remained oligozoospermic (Anderson et al, 1996). The variation in response to testosterone enanthate has been suggested to be related to intrinsic differences in 5 α -reductase activity (Anderson et al, 1996; Anderson et al, 1997) possibly owing to polymorphisms in the 5 α -reductase gene (Elzanaty et al, 2006).

According to public genome databases, more than 300 SNPs mostly in non-coding regions have been detected in the *SRD5A2* gene, but only a few of these have been functionally validated. The most thoroughly examined polymorphism is a C to G transversion at codon 89. The G-allele (L) of V89L (SNP2 in the present study) has been shown to cause reduced activity of the SRD5A2 enzyme *in vitro* (Makridakis et al, 2000). The prevalence of the L-allele is different among ethnic groups being particularly common in Asian populations where the LL-genotype has been found to be associated with approximately 30% lower SRD5A2 activity (Makridakis et al, 1997). However, in men of European origin the association between the V89L polymorphism and

SRD5A2 activity was insignificant (Febbo et al, 1999; Allen et al, 2001; Hayes et al, 2007) and no conclusive evidence of V89L polymorphism affecting serum hormone levels is present (Ntais et al, 2003). Given that SRD5A2 is abundantly expressed in the adult prostate (Levine et al, 1996), the V89L polymorphism has been extensively examined in relation to prostate cancer (e.g. Loukola et al, 2004; Giwercman et al, 2005; Lindstrom et al, 2007). However, recent large studies do not support associations between V89L and the risk of prostate cancer (Pearce et al, 2002; Hayes et al, 2007).

To date, only one study has investigated the influence of *SRD5A2* polymorphisms on sperm parameters. In this study, no correlation between V89L polymorphism and sperm count was found among healthy Caucasians but individuals carrying AT-genotype of the polymorphism A49T exhibited markedly higher sperm concentration compared to AA homozygotes (Elzanaty et al, 2006). Our results are concordant as we did not detect significant differences in V89L genotype distribution between infertile patients and controls, nor between genotypes and sperm count among normozoospermic men. Here, however, we did not examine A49T variation, since the frequency of the T-allele is too low to be used in tagSNP selection.

Spermatozoa acquire progressive motility during migration through the epididymis (reviewed in Cornwall 2009). Normal epididymal functions are dependent upon DHT (Robaire and Viger 1995). SRD5A2 is expressed abundantly throughout the adult epididymis (Mahony et al, 1998), and seminal DHT is of primarily epididymal origin (Anderson et al, 1997). Furthermore, the examination of epididymal sperm maturation following treatment with dual 5 α -reductase inhibitor in rats has been shown to reduce the number of the motile sperm (Henderson and Robaire 2005), thus indicating a role of SRD5A2 activity in gaining sperm motility. On the contrary, reduction of SRD5A2 activity with finasteride failed to affect semen parameters other than seminal volume in young men (Overstreet et al, 1999, Steers 2001). The lack of effect on semen parameters has been proposed to be caused by SRD5A1 activity in germinal epithelium (Steers 2001). Nonetheless, a recent study using of finasteride and dutasteride revealed a small but significant reduction on semen volume and sperm motility in normal men during treatment and follow-up (Amory et al, 2007). Thus, variation in the *SRD5A2* gene may influence sperm motility parameters. The only study exploring the association between *SRD5A2* polymorphisms and sperm motility parameters was conducted within a Swedish population (Elzanaty et al, 2006). Accordingly, the V-allele of the V89L confers a higher proportion of progressive (motility categories A and B) and rapidly progressive (category A) motile spermatozoa than the minor L-

variant. Surprisingly, we found an inverse association between the *SRD5A2* genotypes and sperm motility. Our results suggest that the minor allele of all investigated SNPs (including the L-allele of V89L but not SNP5) was associated with a small but marked rise in the proportion of progressively motile spermatozoa and a lower proportion of immotile spermatozoa among normozoospermic men. In some cases, however, the male pseudohermaphrodites with inherited 5α -reductase type 2 enzyme deficiency and thereby decreased DHT production maintain normal sperm concentration and motility (Cai et al, 1994), suggesting that lower enzyme activities and DHT concentrations affect semen parameters dependent on prostate functions, like semen volume, but may be sufficient for normal spermatogenesis. Still, our results should be interpreted with caution, as there is no clear mechanism explaining how genetic variants associated with moderately decreased enzyme activity or intronic polymorphisms with unknown function would lead to an increased proportion of progressive sperm motility.

Testicular size correlates with testicular function and fertility, being predictive of sperm count and motility as well as serum levels of reproductive hormones (FSH, luteinizing hormone and T) (Takahara et al, 1987). Our results suggest that there is an association between the SNP1 C-allele (GC and CC genotypes) and SNP5 T-allele (CT and TT genotypes) with smaller and larger testicular volume, respectively. The C-allele of SNP1 and the T-allele of SNP5 were over- and underrepresented, respectively, among our infertile patients' group, though these findings were not statistically significant. However, the main androgenic steroid in the testicular environment is testosterone as its local concentration is much higher than that of DHT (Zhao et al, 2004). Therefore, further studies are warranted to confirm the validity of our results.

We did not find any relationship between *SRD5A2* allelic variants and serum levels of reproductive hormones among controls. These results are consistent with previous findings (Elzanaty et al, 2006). In that study authors supposed that the studied polymorphisms did not affect DHT level in serum. Hence, it is unlikely that these polymorphisms also influence the concentrations of other reproductive hormones. Moreover, it has also been shown that the treatment of healthy men with 5α -reductase inhibitors causes no significant changes in their serum gonadotropin levels (Overstreet et al, 1999, Amory et al, 2007).

Conclusion

This is the first case-control study, to our knowledge, that has examined the role of the *SRD5A2* polymorphisms in development of idiopathic male infertility. Our data suggest that common genetic variations in the *SRD5A2* gene are not related to the occurrence of idiopathic azoospermia and oligozoospermia in the Estonian population. Nevertheless, a moderate increase in the proportion of progressively motile spermatozoa was found in normozoospermic men carrying at least one minor allele of all but one of the polymorphisms investigated.

References

- Aljanabi SM, Martinez I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Res.* 1997;25:4692-4693.
- Allen NE, Forrest MS, Key TJ. The association between polymorphisms in the CYP17 and 5alpha-reductase (*SRD5A2*) genes and serum androgen concentrations in men. *Cancer Epidemiol Biomarkers Prev.* 2001;10:185-189.
- Amory JK, Wang C, Swerdloff RS, Anawalt BD, Matsumoto AM, Bremner WJ, Walker SE, Haberer LJ, Clark RV. The effect of 5alpha-reductase inhibition with dutasteride and finasteride on semen parameters and serum hormones in healthy men. *J Clin Endocrinol Metab.* 2007;92:1659-1665.
- Anderson RA, Kelly RW, Wu FC. Comparison between testosterone enanthate-induced azoospermia and oligozoospermia in a male contraceptive study. V. Localization of higher 5 alpha-reductase activity to the reproductive tract in oligozoospermic men administered supraphysiological doses of testosterone. *J Androl.* 1997;18:366-371.
- Anderson RA, Wallace AM, Wu FC. Comparison between testosterone enanthate-induced azoospermia and oligozoospermia in a male contraceptive study. III. Higher 5 alpha-reductase activity in oligozoospermic men administered supraphysiological doses of testosterone. *J Clin Endocrinol Metab.* 1996;81:902-908.
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005;21:263-265.
- Berthaut I, Mestayer C, Portois MC, Cussenot O, Mowszowicz I. Pharmacological and molecular evidence for the expression of the two steroid 5 alpha-reductase isozymes in normal and hyperplastic human prostatic cells in culture. *Prostate.* 1997;32:155-163.
- Cai LQ, Fratianni CM, Gautier T, Imperato-McGinley J. Dihydrotestosterone regulation of semen in male pseudohermaphrodites with 5 alpha-reductase-2 deficiency. *J Clin Endocrinol Metab.* 1994;79:409-414.
- Can S, Zhu YS, Cai LQ, Ling Q, Katz MD, Akgun S, Shackleton CH, Imperato-McGinley J. The identification of 5 alpha-reductase-2 and 17 beta-hydroxysteroid dehydrogenase-3 gene defects in male pseudohermaphrodites from a Turkish kindred. *J Clin Endocrinol Metab.* 1998;83:560-569.
- Chavez B, Valdez E, Vilchis F. Uniparental disomy in steroid 5alpha-reductase 2 deficiency. *J Clin Endocrinol Metab.* 2000;85:3147-3150.

- Cornwall GA. New insights into epididymal biology and function. *Hum Reprod Update*. 2009;15:213-227.
- de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet*. 2005;37:1217-1223.
- Eicheler W, Tuohimaa P, Vilja P, Adermann K, Forssmann WG, Aumuller G. Immunocytochemical localization of human 5 alpha-reductase 2 with polyclonal antibodies in androgen target and non-target human tissues. *J Histochem Cytochem*. 1994;42:667-675.
- Elzanaty S, Giwercman YL, Giwercman A. Significant impact of 5alpha-reductase type 2 polymorphisms on sperm concentration and motility. *Int J Androl*. 2006;29:414-420.
- Febbo PG, Kantoff PW, Platz EA, Casey D, Batter S, Giovannucci E, Hennekens CH, Stampfer MJ. The V89L polymorphism in the 5alpha-reductase type 2 gene and risk of prostate cancer. *Cancer Res*. 1999;59:5878-5881.
- Fernandez-Cancio M, Nistal M, Gracia R, Molina MA, Tovar JA, Esteban C, Carrascosa A, Audi L. Compound heterozygous mutations in the SRD5A2 gene exon 4 in a male pseudohermaphrodite patient of Chinese origin. *J Androl*. 2004;25:412-416.
- Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D. The structure of haplotype blocks in the human genome. *Science*. 2002;296:2225-2229.
- Giwercman YL, Abrahamsson PA, Giwercman A, Gadaleanu V, Ahlgren G. The 5alpha-reductase type II A49T and V89L high-activity allelic variants are more common in men with prostate cancer compared with the general population. *Eur Urol*. 2005;48:679-685.
- Hayes VM, Severi G, Padilla EJ, Morris HA, Tilley WD, Southey MC, English DR, Sutherland RL, Hopper JL, Boyle P, Giles GG. 5alpha-Reductase type 2 gene variant associations with prostate cancer risk, circulating hormone levels and androgenetic alopecia. *Int J Cancer*. 2007;120:776-780.
- Henderson NA, Robaire B. Effects of PNU157706, a dual 5alpha-reductase inhibitor, on rat epididymal sperm maturation and fertility. *Biol Reprod*. 2005;72:436-443.
- Hines RS, Tho SP, Behzadian MA, McDonough PG. Steroid 5alpha-reductase 2 gene melting polymorphisms in male subjects with azoospermia or oligospermia. *Am J Obstet Gynecol*. 1999;180:1394-1398.
- Imperato-McGinley J, Zhu YS. Androgens and male physiology the syndrome of 5alpha-reductase-2 deficiency. *Mol Cell Endocrinol*. 2002;198:51-59.
- Jenkins EP, Hsieh CL, Milatovich A, Normington K, Berman DM, Francke U, Russell DW. Characterization and chromosomal mapping of a human steroid 5 alpha-reductase gene and pseudogene and mapping of the mouse homologue. *Genomics*. 1991;11:1102-1112.
- Jensen TK, Jorgensen N, Punab M, Haugen TB, Suominen J, Zilaitiene B, Horte A, Andersen AG, Carlsen E, Magnus O, Matulevicius V, Neramo I, Vierula M, Keiding N, Toppari J, Skakkebaek NE. Association of in utero exposure to maternal smoking with reduced semen quality and testis size in adulthood: a cross-sectional study of 1,770 young men from the general population in five European countries. *Am J Epidemiol*. 2004;159:49-58.
- Johnson GC, Esposito L, Barratt BJ, Smith AN, Heward J, Di Genova G, Ueda H, Cordell HJ, Eaves IA, Dudbridge F, Twells RC, Payne F, Hughes W, Nutland S, Stevens H, Carr P,

- Tuomilehto-Wolf E, Tuomilehto J, Gough SC, Clayton DG, Todd JA. Haplotype tagging for the identification of common disease genes. *Nat Genet.* 2001;29:233-237.
- Labrie F, Sugimoto Y, Luu-The V, Simard J, Lachance Y, Bachvarov D, Leblanc G, Durocher F, Paquet N. Structure of human type II 5 alpha-reductase gene. *Endocrinology.* 1992;131:1571-1573.
- Levine AC, Wang JP, Ren M, Eliashvili E, Russell DW, Kirschenbaum A. Immunohistochemical localization of steroid 5 alpha-reductase 2 in the human male fetal reproductive tract and adult prostate. *J Clin Endocrinol Metab.* 1996;81:384-389.
- Lindstrom S, Adami HO, Balter KA, Xu J, Zheng SL, Stattin P, Gronberg H, Wiklund F. Inherited variation in hormone-regulating genes and prostate cancer survival. *Clin Cancer Res.* 2007;13:5156-5161.
- Loukola A, Chadha M, Penn SG, Rank D, Conti DV, Thompson D, Cicek M, Love B, Bivolarevic V, Yang Q, Jiang Y, Hanzel DK, Dains K, Paris PL, Casey G, Witte JS. Comprehensive evaluation of the association between prostate cancer and genotypes/haplotypes in CYP17A1, CYP3A4, and SRD5A2. *Eur J Hum Genet.* 2004;12:321-332.
- Mahony MC, Swanlund DJ, Billeter M, Roberts KP, Pryor JL. Regional distribution of 5alpha-reductase type 1 and type 2 mRNA along the human epididymis. *Fertil Steril.* 1998;69:1116-1121.
- Makridakis N, Ross RK, Pike MC, Chang L, Stanczyk FZ, Kolonel LN, Shi CY, Yu MC, Henderson BE, Reichardt JK. A prevalent missense substitution that modulates activity of prostatic steroid 5alpha-reductase. *Cancer Res.* 1997;57:1020-1022.
- Makridakis NM, di Salle E, Reichardt JK. Biochemical and pharmacogenetic dissection of human steroid 5 alpha-reductase type II. *Pharmacogenetics.* 2000;10:407-413.
- Mazen I, Gad YZ, Hafez M, Sultan C, Lumbroso S. Molecular analysis of 5alpha-reductase type 2 gene in eight unrelated egyptian children with suspected 5alpha-reductase deficiency: prevalence of the G34R mutation. *Clin Endocrinol (Oxf).* 2003;58:627-631.
- Montpetit A, Nelis M, Laflamme P, Magi R, Ke X, Remm M, Cardon L, Hudson TJ, Metspalu A. An evaluation of the performance of tag SNPs derived from HapMap in a Caucasian population. *PLoS Genet.* 2006;2:e27.
- Ntais C, Polycarpou A, Ioannidis JP. SRD5A2 gene polymorphisms and the risk of prostate cancer: a meta-analysis. *Cancer Epidemiol Biomarkers Prev.* 2003;12:618-624.
- Overstreet JW, Fuh VL, Gould J, Howards SS, Lieber MM, Hellstrom W, Shapiro S, Carroll P, Corfman RS, Petrou S, Lewis R, Toth P, Shown T, Roy J, Jarow JP, Bonilla J, Jacobsen CA, Wang DZ, Kaufman KD. Chronic treatment with finasteride daily does not affect spermatogenesis or semen production in young men. *J Urol.* 1999;162:1295-1300.
- Payne AH, Kawano A, Jaffe RB. Formation of dihydrotestosterone and other 5 alpha-reduced metabolites by isolated seminiferous tubules and suspension of interstitial cells in a human testis. *J Clin Endocrinol Metab.* 1973;37:448-453.
- Pearce CL, Makridakis NM, Ross RK, Pike MC, Kolonel LN, Henderson BE, Reichardt JK. Steroid 5-alpha reductase type II V89L substitution is not associated with risk of prostate cancer in a multiethnic population study. *Cancer Epidemiol Biomarkers Prev.* 2002;11:417-418.

- Robaire B, Viger RS. Regulation of epididymal epithelial cell functions. *Biol Reprod.* 1995;52:226-236.
- Ross RK, Pike MC, Coetzee GA, Reichardt JK, Yu MC, Feigelson H, Stanczyk FZ, Kolonel LN, Henderson BE. Androgen metabolism and prostate cancer: establishing a model of genetic susceptibility. *Cancer Res.* 1998;58:4497-4504.
- Steers WD. 5alpha-reductase activity in the prostate. *Urology.* 2001;58:17-24.
- Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet.* 2001;68:978-989.
- Zhao M, Baker SD, Yan X, Zhao Y, Wright WW, Zirkin BR, Jarow JP. Simultaneous determination of steroid composition of human testicular fluid using liquid chromatography tandem mass spectrometry. *Steroids.* 2004;69:721-726.
- Takahara H, Cosentino MJ, Sakatoku J, Cockett AT. Significance of testicular size measurement in andrology: II. Correlation of testicular size with testicular function. *J Urol.* 1987;137:416-419.
- Thigpen AE, Davis DL, Milatovich A, Mendonca BB, Imperato-McGinley J, Griffin JE, Francke U, Wilson JD, Russell DW. Molecular genetics of steroid 5 alpha-reductase 2 deficiency. *J Clin Invest.* 1992;90:799-809.
- Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD, Russell DW. Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression. *J Clin Invest.* 1993;92:903-910.
- WHO. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. 4th ed. Cambridge, UK, Cambridge University Press. 1999.
- Vilchis F, Mendez JP, Canto P, Lieberman E, Chavez B. Identification of missense mutations in the SRD5A2 gene from patients with steroid 5alpha-reductase 2 deficiency. *Clin Endocrinol.* 2000;52:383-387.

Figure legend

Figure. Schematic structure of the *SRD5A2* gene with locations of genotyped tagSNPs indicated and linkage disequilibrium plot displaying D' values (%) for each pair of SNPs. The haplotype block is indicated. The plot was generated using Haploview 4.0 software and a confidence intervals algorithm (Gabriel et al, 2002).

Table 1. The primer sequences and restriction enzymes used for *SRD5A2* genotyping

Polymorphism	Primer sequences (5' - 3')*	Restriction enzyme	Allele	DNA fragment size (bp)
SNP1 (rs632148)	F-GAGAGCGCGGCCCCCGCAAG <u>C</u> R-GGCCTTCGTTCTCCTCCGGCCA	<i>Eco47III</i>	C G	180 158+22
SNP2 (rs523349)	F- GTGAAGGCGGCGTCTGTG R- TCGGGCCACCTGGGACGCTA	<i>RsaI</i>	G C	275 235+40
SNP3 (rs2300701)	F-ATCAACTTGAGGTGGATTTTGGGATC R-GCTGCTCTTCATAGGCTTGCTGA	<i>PagI</i>	G A	148 126+22
SNP4 (rs2268797)	F-CACTCCAATGTTTGCCTCTGTC R-TCATATATTTAGTGTGCCATTGA <u>TC</u>	<i>BclI</i>	C T	190 166+24
SNP5 (rs12470143)	F-GTTTATTCTTCCAATTGCTTAAGC R-AATAGATTGCGGTGGAGTA <u>TACGT</u>	<i>Eco105I</i>	C T	251 227+24

*F and R indicate, respectively, forward and reverse primers. The nucleotides modified to create specific restriction sites are underlined.

Table 2. Distribution of *SRD5A2* gene genotypes in controls and infertile patients*

Polymorphism	Genotype	Controls n (%)	Patients n (%)	P value
SNP1	GC+CC	101 (47.9)	77 (58.3)	0.070
rs632148	GG	110 (52.1)	55 (41.7)	
SNP2 (V89L)	CG (VL)+GG (LL)	100 (47.4)	74 (56.1)	0.126
rs523349	CC (VV)	111 (52.6)	58 (43.9)	
SNP3	GA+AA	128 (60.7)	91 (68.9)	0.110
rs2300701	GG	83 (39.3)	41 (31.1)	
SNP4	TC+CC	134 (63.5)	92 (69.7)	0.282
rs2268797	TT	77 (36.5)	40 (30.3)	
SNP5	CT+TT	151 (71.6)	87 (65.9)	0.290
rs12470143	CC	60 (28.4)	45 (34.1)	

*Statistical analysis was performed using χ^2 test.

Table 3. Distribution of *SRD5A2* gene major haplotypes (> 5% prevalence) in controls and infertile patients*

Haplotype	Frequency %			P value
	All	Controls	Patients	
1 GCGTT	39.0	41.4	35.2	0.106
2 CGACC	25.9	24.0	28.8	0.165
3 GCGCC	10.4	11.7	8.5	0.181
4 GCATC	6.6	6.7	6.5	0.937

*Statistical analysis was performed using χ^2 test.

Table 4. Correlations between sperm motility parameters, testicular volume and *SRD5A2* genotypes among control group men*

Variables	SNP1		SNP2		SNP3		SNP4		SNP5	
	GG n = 108	GC+CC n = 101	CC n = 109	CG+GG n = 100	GG n = 81	GA+AA n = 128	TT n = 75	TC+CC n = 134	CC n = 60	CT+TT n = 149
Sperm motility										
A+B [†] %	56.1 (9.6)	59.6 (9.9) [‡]	56.1 (9.7)	59.6 (9.9) [‡]	55.5 (9.2)	59.2 (10.0) [‡]	55.5 (8.7)	59.1 (10.0) [‡]	58.0 (10.0)	57.7 (9.5)
C %	13.0 (4.8)	12.5 (4.3)	13.0 (4.8)	12.5 (4.4)	12.9 (4.8)	12.6 (4.4)	12.7 (4.2)	12.8 (4.8)	12.7 (5.0)	12.8 (4.4)
D %	30.9 (8.8)	27.8 (9.2) [‡]	30.8 (8.8)	27.8 (9.1) [‡]	31.5 (9.0)	28.0 (8.9) [‡]	31.7 (8.2)	28.0 (9.3) [‡]	29.2 (9.6)	29.5 (8.9)
Testicular volume (mL)	27.3 (4.7)	25.8 (5.0) [‡]	27.2 (4.7)	26.0 (5.0)	27.1 (5.0)	26.3 (4.8)	27.2 (4.9)	26.2 (4.9)	25.4 (4.8)	27.0 (4.9) [‡]

*Homozygous wild-type genotypes were compared with combined genotypes (heterozygotes and variant homozygotes) separately for different sperm motility categories. Statistical analysis was performed using linear regression model adjusted by the length of abstinence and age, values are given as mean (\pm SD).

[†]A+B = progressively motile spermatozoa.

[‡]P < 0.05

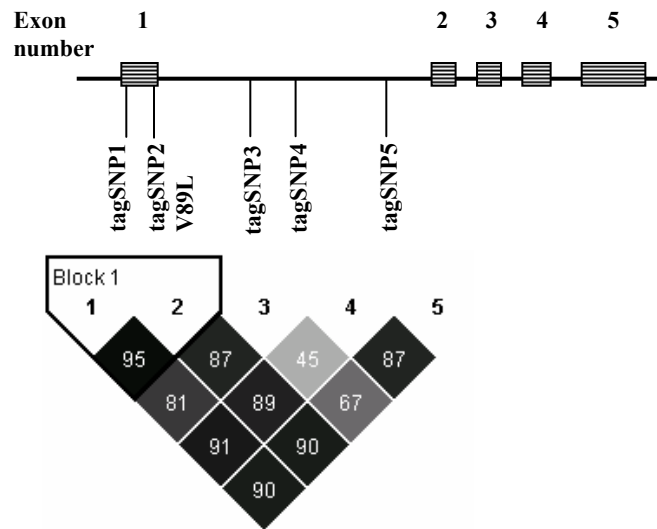


Figure 1, Peters et al.